 doi:10.20944/preprints202102.0390.v1

Type of the Paper (Article)

Bioassay-guided isolation of 2-[p-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol from *Oroxylum indicum* and investigation of its molecular mechanism action of apoptosis induction

Asem Robinson Singh[1], Salam Asbin Singh[1], Thangjam Davis Singh[1], Naorem Tarundas Singh[1], Takhellambam Chanu Machathoibi[1], Okram Mukherjee Singh[2], Lisam Shanjukumar Singh[1]*

1 *Cancer and Molecular Biology Division, Department of Biotechnology, Manipur University, Canchipur, Imphal-795003, Manipur, India*
2 *Department of Chemistry, Manipur University, Canchipur, Imphal-795003, Manipur, India*
* Correspondence: shanju.lisam@manipuruniv.ac.in; Tel.: +91-385-2435233; Mobile: 91-9615533300

Abstract: Leaf crude extract (aqueous) of *Oroxylum indicum* (L.) Kurz induces genomic DNA fragmentation, comet formation, and inhibition of cell proliferation in prostate cancer cell line, PC3 as assessed by agarose gel electrophoresis, comet assay, and MTT assay respectively. The bioactive compound was purified through bioassay-guided fractionation using preparative HPLC and MTT assay. The brown and water-soluble compound was characterized using 1H and 13C nuclear magnetic resonance (NMR), fourier transform infrared (FT-IR) and electrospray ionization (ESI) mass spectrometry, and the compound was identified as a glycosylated hydroquinone derivative, 2-[p-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol (molecular formula, $C_{13}H_{18}N_2O_8$; molecular mass = 330). The identified phytocompound has not been reported earlier elsewhere. Therefore, the common name of the novel anticancer phytocompound isolated from *oroxylum indicum* in this current study is named as oroxyquinone. The half-maximal inhibitory concentration (IC_{50}) of oroxyquinone on PC3 cells was 19.44 µg/ml (95% CI = 17.97 to 21.04). Oroxyquinone induced cell cycle arrest at S phases and inhibition of cell migration on PC3 as assessed by flow cytometry and wound healing assay respectively. On investigating the molecular mechanism of inducing apoptosis, the results indicated that the oroxyquinone induced apoptosis through the p38 pathway and cell cycle arrest, however, not through caspase-3 and PARP pathways. The present study identifies a novel anticancer molecule and provides scientific evidence supporting the therapeutic potency of OI for ethnomedicinal uses.

Keywords: Oroxylum indicum, Oroxyquinone, Traditional medicine, Bioassay Guided Fractionation, caspase-independent apoptosis, anti-metastatic.

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1. Introduction

Oroxylum indicum (L.) Kurz (Kingdom-Plantae, Class-Magnoliophyta, Order- Lamiales, Family- Bignoniaceae, genus-Oroxylum, species-Indicum, local name *shamba, in Manipuri*) is found throughout the Asian sub-continent including India. *Oroxylum indicum* (OI) has been in use in many folk medicines of India [1,2], Bangladesh [3], Vietnam[4], China [5] and Thailand [6] for the treatment of cancer, gastric ulcer, fever, arthritis, etc.[2,7-13]. In the recent past, several studies have been undertaken on OI for their phytochemical properties and pharmacological activities and suggested anti-cancer, free radical scavenging, immune-stimulant effects of OI. Costa-Lotufo et al., 2005 reported cytotoxic activity of stem bark extract of OI on B-16 and HL-60 [3], more recently in HeLa by Moirangthem et al., 2013 [14]. The antimutagenic property of the plant extract has also been described by Nakahara et al. [15]. Naveen Kumar et al, described the anticancer activity of stem bark extract of the plant in breast cancer cell lines MDA-MB-231 with petroleum extract exhibiting the highest activity [16]. However, a detailed mechanism of the anticancer mechanism or any evidence of antiapoptotic activity of the plant extract has not been reported yet. In an attempt to isolate all the present flavonoids in the seed of OI, Kruger and Ganzera have isolated six major flavonoids including baicalein, chrysin, oroxylin, and their derivatives recently [17]. Baicalein has been shown to associate with the accumulation of cells at S or G2M phases. The results indicated that baicalein has antitumor effects on human cancer cells. Recently, it is reported that oroxin A which is also known as baicalein-7-O-glucoside isolated from OI prevents the progression from prediabetes to diabetes in streptozotocin and high-fat diet-induced mice [18]. More recently study on the anticancer activity of the plant has been of immense interest and many studies have been carried out. An attempt to purify bioactive phytocompound with anticancer activities on breast cancer cells was tried by Rajkumar V and colleagues, however, the molecular mechanism of cell death associated with the compound was not studied [19]. Anticancer activity of crude extract of the plant is further supported by many studies [8,14,16,20]. Bicalein, isolated from the fruits of OI has been shown to induce apoptosis in HL-60 cell apoptosis as assessed Tunnel assay and DNA fragmentation, however, the molecular mechanism was not elucidated [21].

Cancer ranks as one of the leading causes of mortality and morbidity worldwide with most cases seen in developed nations. It has a mortality rate of one in eight deaths worldwide which is a figure that surpasses the cumulative death rate of AIDS, tuberculosis as well as malaria [22]. According to the World Health Organization (WHO), in 2018, 18.1 million people around the world had cancer, and 9.6 million died from the disease. By 2040, those figures will nearly double, with the greatest increase in low-income countries, where more than two-thirds of the world's cancers will occur. Cancer is the cause of about 30% of all premature deaths from non-communicable diseases among adults aged 30-69. The total number of cancers diagnosed in India is between the years 2017 and 2018 is 7,84,821 (an increase of 324%) [23].

The high prevalence of cancer in the more developed countries is believed to be associated with dietary habits as well as the lifestyle of the

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people. Among men, the most common cancer that has significant mortality and morbidity are prostate, colorectal, liver, stomach and lung cancer. With an expected rise of more than 70% of new cases in the coming two decades finding a solution to the medical problem is a concern for health care organizations around the globe. Chemotherapeutic treatment of cancer is often associated with side effects resulting from toxicities of the drug. In cancer prevention and treatment, traditional medicines have played an important role for centuries. A few of the plant-derived anticancer agents that are in use today for the management of cancer are alkaloids derived from *Vinca rosea*, taxanes from *Taxus brevifolia*, camptothecin derived drugs from *Camptotheca acuminata,* and epipodophyllotoxins of *Podophyllum peltatum*. And yet a huge potential lies ahead to discovering new phytochemicals that can be used as chemotherapeutic drugs. The current study aims to purify and identify anti-cancer phytocompound from the leaf of OI through Bioassay-Guided Fractionation and to investigate its molecular mechanism.

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2. Results

2.1. Leaf crude extract of OI inhibits the proliferation of PC3 cells

PC3 cells were treated with different doses of extract for different time points. The results showed that there was significant inhibition of cell proliferation in a dose and time-dependent manners (Fig 1A). Therefore, the results indicate that the leaf extract of OI has cytotoxic activity towards PC3 cells. Further IC_{50} of the leaf extract was determined by treating the cells with different doses of the extract for 24 hrs and cell viability was determined by MTT assay as above. The result also showed that IC_{50} value of the leaf crude extract of OI was 69.99 µg/ml (95% CI = 57.11 to 85.77) (Fig 1 A & B).

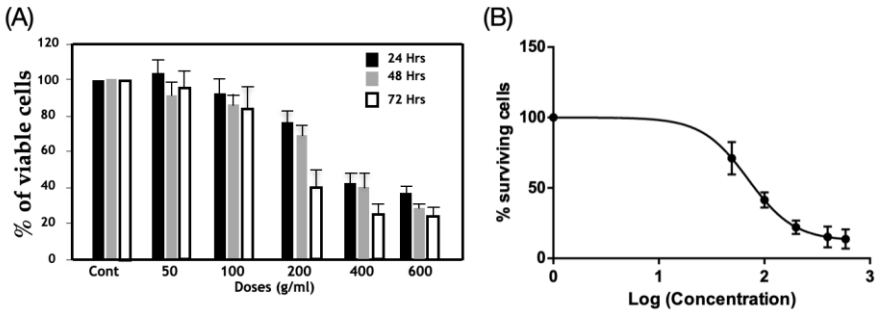

Figure 1. Leaf crude extract of OI inhibits proliferation of PC3: (A) Leaf crude extract of OI was treated to PC3 at different doses (50-, 100-, 200-, 400- and 600 g/ml) for 24-, 48- and 72 hrs and effect on cells proliferation was assessed by MTT assays. **(B)** The half-maximal inhibitory concentration (IC_{50}) of leaf crude extract for inhibition of cell proliferation was measured on PC3 cells by treating the cells with the doses as above for 48 hrs and MTT assays were conducted. Control (Cont) cells were treated with DMSO under identical conditions as treated cells. IC_{50} was measured using GraphPad Prism 8 software. The percentage of viable cell numbers of each treated group was calculated after taking the viable numbers of control cells as 100%. P-values were calculated using the student's "*t*" test. Bars indicates mean ±SD, * indicates p-value > 0.05, ** indicates p-value > 0.01 and *** indicates p-value > 0.001.

2.2 The leaf crude extract of OI induces apoptosis in PC3 cells

To further investigate the mechanism of inhibition of cell proliferation induced by the leaf crude extract of OI, gDNA fragmentation (DNA ladder formation) which is the well-established hallmark of cell apoptosis was analyzed as described in materials and methods in PC3 cells. The results showed that the leaf extract induced gDNA fragmentation in PC3 cells at 24 hrs treatment in a dose-dependent manner (Fig 2A). The finding was further confirmed by comet assay which is based on single-cell genomic DNA fragmentation as described in materials and methods (Fig 2B). The results indicated that in PC3 cells, even at a low dose (50µg/ml) gDNA fragmentation was observed and the length of the comet tails increased with the dose of the leaf extract (Fig 2C). The result of inhibition of cell proliferation is in accordance with the results of DNA fragmentation. Therefore, the results clearly indicated that the crude leaf extract of OI inhibits cell proliferation through induction of program cell death, apoptosis.

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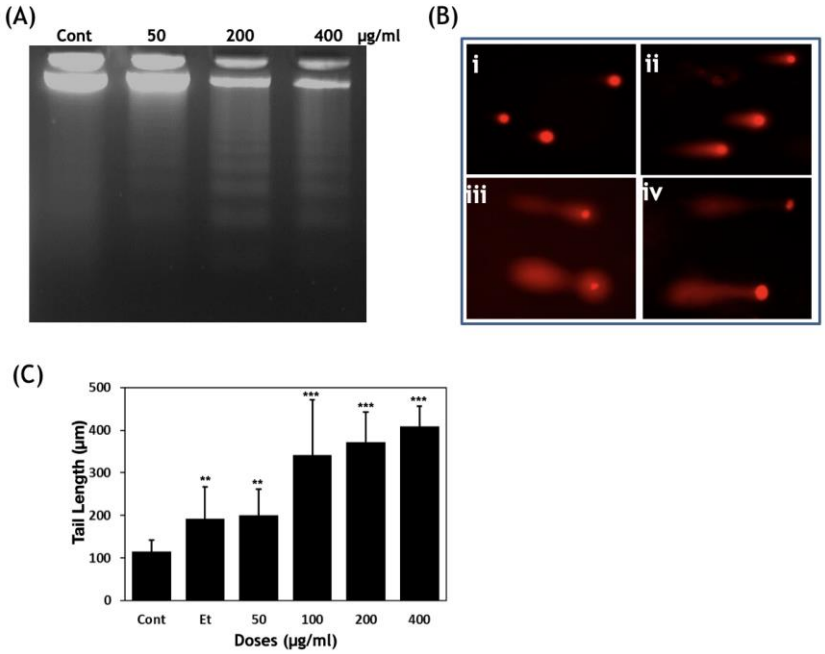

Figure 2. Leaf extract of OI triggers gDNA fragmentation in PC3 cells: PC3 cells were treated with 50, 100, 200, or 400µg/ml of leaf extract dissolved in 10µl DMSO for 24 h. Negative control cells (Cont) were treated with 10µl DMSO. After treatment **(A)** DNA ladder formation and **(B)** comet assays were conducted as described in materials and methods; (i) Control, (ii) 50µg/ml, (iii) 200µg/ml (iv) and 400µg/ml. **(C)** The tail length of 25 comets in each group was measured and mean and standard deviation were calculated. P-values were calculated for each group comparing with the control group using the student's "*t*" test. ** indicates p-values >0.05 and *** p-values >0.001. Bars indicate mean ±SD.

2.3 Bioassay Guided Fractionation purified a novel bioactive compound

To purify the bioactive compound of the leaf of OI that induces cell apoptosis, a Bioassay Guided Fractionation (BGF), employing silica column chromatography, preparative HPLC, and high throughput MTT assay was used as described in materials and methods. Twelve fractions of 50ml each collected from silica column chromatography were assessed for inhibition of cell proliferation by MTT assays. The third and fourth fractions out of 12 obtained from silica column chromatography showed inhibition of cell proliferation of PC3 whereas other fractions showed no activity (data not shown). Both fractions from silica column chromatography were further analyzed and fractionated on preparative HPLC using a C-18 column as described in materials and methods. All the major single peaks of the HPLC profiles were collected in 200µl volume using a fraction collector and assessed for inhibition of cell proliferation by MTT assays as above (data not shown). The single peak of 9.02 min retention time obtained from the third fraction was found to possess the bioactivity for inhibition of cell proliferation as assessed by MTT assay and induced cell shrinking as assessed by morphological changes under a light microscope (Fig 3A). We further analyzed the purity of the compound on HPLC. The analyses showed a clear and sharp single peak indicating it is a single compound (Fig 3B). Finally, the activity for apoptosis induction of the purified compound was confirmed by comet assays (data not shown). The IC_{50} of the purified phytocompound for inhibition of cell proliferation was determined on PC3 cells by MTT assays. The results revealed that the purified phytocompound has an IC_{50} value of 19.44 µg/ml (95% CI = 17.97 to 21.04) (Fig 3 C&D).

2.4 Chemical characterization

Brown colored solid and water soluble; 1H NMR (500 MHz, CDCl3, δ ppm): 7.37-7.42 (m, 1H), 8.32 (s, 3H), 6.85 (d, J=8.3 Hz, 2H), 5.99 (d, J=8.3 Hz, 2H), 4.19 (d, J=10.3 Hz, 1H), 3.96 (m, 1H), 3.84 (m, 1H), 3.59 (m, 1H), 2.74 (m, 1H), 2.65 (m, 1H), 2.56 (m, 1H), 2.21

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(m, 4OH); 13C NMR (125.8MHz, CDCl3, δ ppm): 35.7, 39.3, 42.3, 59.5, 66.2, 69.8, 74.8, 80.8, 127.7, 150.1, 139.1, 153.3, 171.1, 200.9; IR (KBr) (ν max, cm−1): 1099.4, 1382.9, 1589.3, 3336.8 cm-1; MS: m/z = 330 (M+). Anal. Calcd for $C_{13}H_{18}N_2O_8$; C, 72.16; H, 3.78; S, 12.04. Found: C, 47.27; H, 5.49; N, 8.48; O, 38.75. On the basis of these observations the purified compound is proposed to be 2-[p-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol. The chemical structure elucidated by ChemDoodle Software (iChemLabs, Ver. 9.0.3) is shown in fig. 4. Analysis of 2-[p-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol using chemical databases namely SciFinder, ChemSpider, Pubchem and Pubmed revealed that the purified phytocompound is not found in the these chemical databases, indicating it is a novel compound. Therefore, the common name of this novel phytochemical is given as Oroxyquinone for convenience.

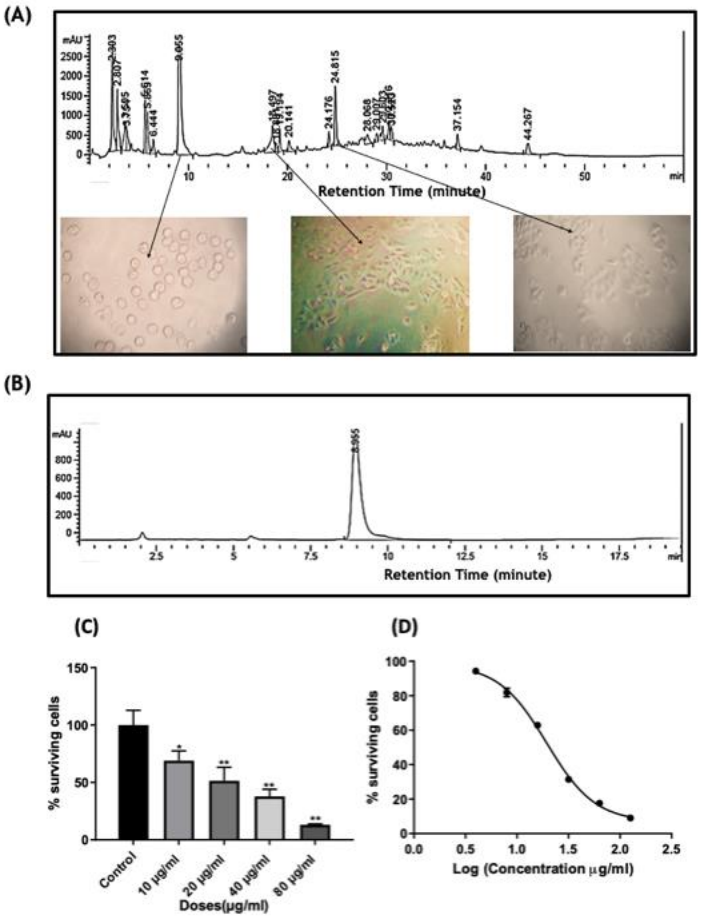

Figure 3. Purification of the bioactive compound using HPLC. Leaf extract was subjected to silica column chromatographic fractionation and then each fraction was subjected to bioassay as described in materials and methods. **(A)** Fraction 3 of the silica column chromatography was further subjected to preparative HPLC fractionation and single peaks were further subjected to bioassay. **(B)** A single peak of 9.052 min retention time showed anticancer activity and it was further analyzed on HPLC for purity. **(C)** The purified phytocompound collected as a single peak on HPLC was subjected to MTT assay after treating PC3 cells with 10-, 20-, 40- and 80 µg/ml for 24 hrs. The percentage of viable cell numbers of each treated group was calculated after taking the viable numbers of control cells as 100%. P-values were calculated using the student's "*t*" test. Bars indicates mean ±SD, * indicates p-value > 0.05, ** indicates p-value > 0.01 and *** indicates p-value >0.001. **(D)** IC_{50} for the purified phytocompound was measured using GraphPad Prism 8 software.

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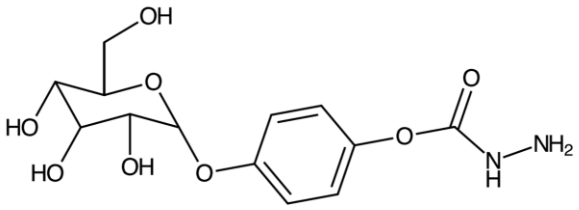

$C_{13}H_{18}N_2O_8$

2-[*p*-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3,4,5-triol

Molecular mass = 330

Figure 4. Chemical structure: After NMR, HRMS, and FT-IR spectra were analyzed, the chemical structure and IUPAC name of the purified phytocompound was generated using ChemDoodle Software (iChemLabs, Ver. 9.0.3).

2.5 Oroxyquinone induces cell cycle arrest in PC3 cells

To further investigate the effect of oroxyquinone on cell division, we conducted a cell cycle analysis in PC3 on a flow cytometer as described in materials and methods. The results showed that oroxyquinone induced cell cycle arrest at S phase in a dose-dependent manner in PC3 cells. The number of PC3 cells at the S phase were 16.6±1.46%, 23.27±2.27%, 28.23 ±2.65% and 36.1±4.75% at 0, 10, 20 and 40µg/ml of oroxyquinone treatment respectively (Fig 5A & B). The number of cells at G_0/G_1 phases decreases with doses of oroxyquinone. However, the number of cells remains almost unchanged at the G_2/M phases. Therefore, the findings clearly indicate that oroxyquinone triggers cell cycle arrest at S phase which may be possibly due to DNA fragmentation induced by the novel phytocompound.

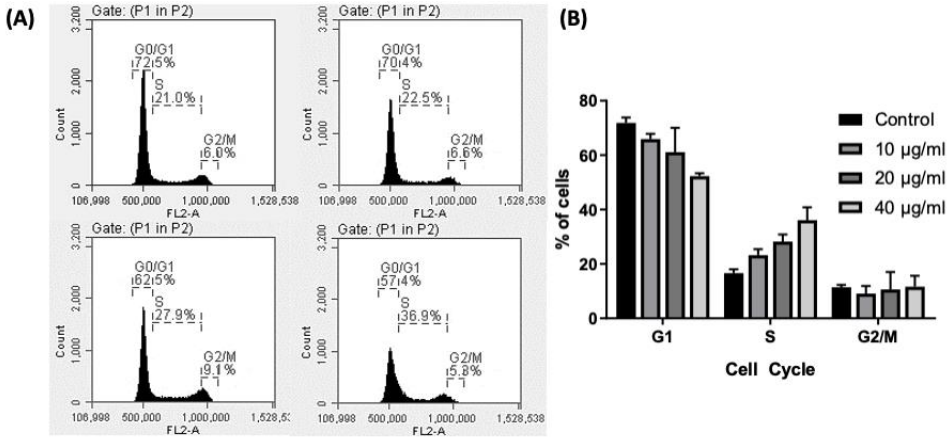

Figure 5. Oroxyquinone triggers cell cycle arrest: Oroxyquinone triggers cell cycle arrest: (A) PC3 cells were cultured and treated with (i) 0µg/ml (Control), (ii) 10µg/ml, (iii) 20µg/ml (iv) 40µg/ml of oroxyquinone as indicated for 16 hrs. **(B)** Cells were subjected to propidium iodide staining to measure DNA content in each cell by flow cytometry. Percentage of cells in phases of cell cycle was compared between control and treated cells. The number of cells in S phase increased with an increase in dose in a statistically significant manner ($p < 0.05$).

2.6 Oroxyquinone inhibits PC3 cell migration

To investigate the anti-metastatic potential of oroxyquinone, wound healing assay was conducted on PC3 which is a highly metastatic cell. Initially, MTT assays were conducted on PC3 cells with low doses of oroxyquinone (data not shown), and 5µg/ml which does not exhibit inhibition of cell proliferation was selected for the wound healing assay. The results showed that oroxyquinone (5µg/ml) significantly reduced the PC3 cell migration compared to control (untreated) cells by approximately 35% about at 6 hrs and approximately 55% at 12 hrs (Fig 6 A&B). The finding strongly suggested that the novel phytochemical, oroxyquinone purified from the leaf of OI has anti-metastatic potency toward highly metastatic cells in addition to its anti-apoptotic potency.

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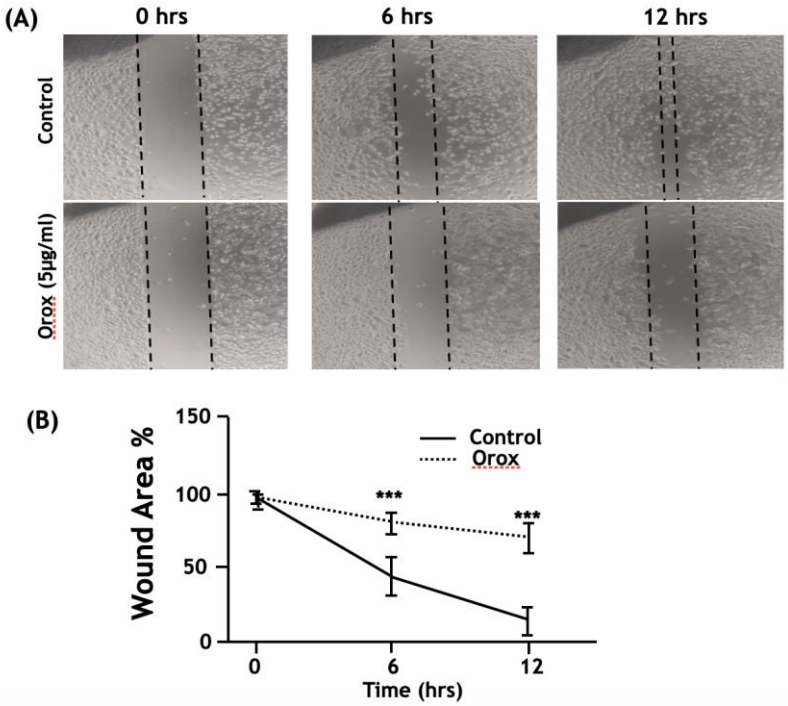

Figure 6. Oroxyquinone inhibits PC3 cell migration: (A) PC3 cells were cultured and wound healing assay was conducted as described in materials and methods by treating with 5µg/ml of oroxyquinone (Orox) or PBS (Control). The pictures represent the images recorded of the same cells of each group recorded at 0, 6, and 12 hrs of treatment. The dark marks on the upper left side of the image were to make sure images were recorded on the same field each time. **(B)** To measure the area of the wound, the length of scratches was measured on the light microscope and plotted on a graph. Wound area of control 0 hr was taken as 100% and mean ±SD. P-values were calculated comparing with the control values of the same hrs of treatment by students' "*t*" test. *** indicates p-value >0.001.

2.7 Oroxyquinone induces cells apoptosis in PC3 cells in a caspase- and PARP-independent manner

To determine the molecular mechanisms of the apoptosis induced by oroxyquinone in PC3 cells, activation of caspase-3 and deactivation of PARP upon treatment were assessed using western blotting. Cleavage of caspase-3 was not observed in PC3 cells even at the highest dose used (40µg/ml) except the positive control (etoposide) (Fig 7 A). Further cleavage of PARP which is the downstream protein of caspase-3 was investigated. As expected, cleavage of PARP was not observed in PC3 cells at any dose used (Fig 7B). Therefore, the results clearly suggested that oroxyquinone induces apoptosis in a caspase- and PARP-independent manner.

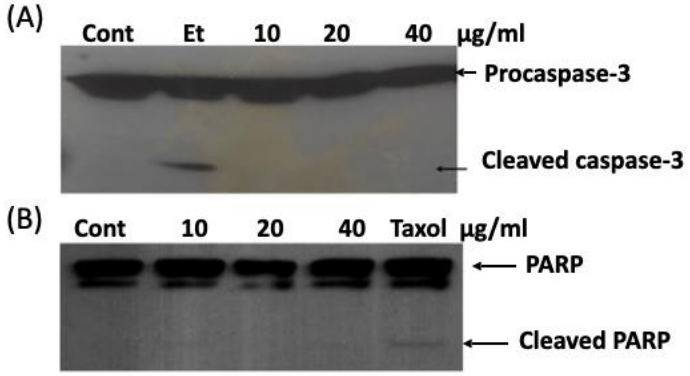

Figure 7. Oroxyquinone induces caspase-3 and PARP independent apoptosis in PC3 cells: PC3 cells were cultured and treated with different doses of oroxyquinone, total proteins extracted and separated by SDS-PAGE and immunoblotted using antibodies against **(A)** procaspase-3 and **(B)** PARP.

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Further, the signaling pathways followed by oroxyquinone for cell apoptosis induction were investigated by western blotting using antibodies against the total as well as phosphorylated ERK, JNK, and p38 proteins. The results showed that in PC3 cells oroxyquinone activates p38 and JNK proteins at all the doses used (10, 20, and 40µg/ml) in a dose-dependent manner (Fig 8, upper & middle panels). However, ERK is not activated at lower doses (10 and 20µg/ml) of oroxyquinone but only at the highest dose (40µg/ml) used (Fig 8, left lower panel). To confirm the functional roles of p38, JNK, and ERK involved in the oroxyquinone induced apoptosis in PC3 cells, MTT assays were conducted in the presence and absence of specific inhibitors of p38, JNK, and ERK. The results revealed that the presence of a specific inhibitor of p38 (SB203580) abrogates the oroxyquinone induced inhibition of PC3 cell proliferation whereas the presence of specific inhibitors for JNK (SP600125) or ERK (U0126) enhances inhibition of PC3 cells proliferation (Fig 8B).

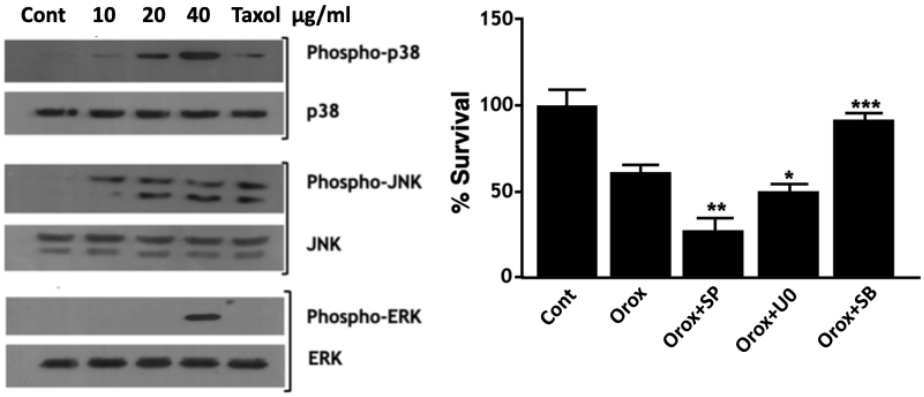

Figure 8. Oroxyquinone induces apoptosis via MARKs pathways: (A) PC3 cells were cultured and treated with different doses of oroxyquinone, total proteins extracted and separated by SDS-PAGE, and immunoblotted using antibodies against total and phosphorylated forms of p38, JNK, and ERK. **(B)** PC3 cells were cultured and treated with PBS as control (Cont), IC_{50} of oroxyquinone (Orox) or with specific inhibitors SP600125 (SP), U0126 (U0), and SB203580 (SB) for JKN, ERK, and p38 respectively for 24 hrs, and MTT assays were performed. P-value was calculated comparing to the oroxyquinone treated cells using the student's "t" test. Bars indicates mean ±SD, * indicates p-value > 0.05, ** indicates p-value > 0.01 and *** indicates p-value >0.001.

3. Discussion

The current study has shown that the crude extract of leaf of OI has potent anti-proliferative activity in PC3 cells in a time and dose-dependent manner with an IC_{50} value of 69.99 µg/ml (95% CI = 57.11 to 86.77) as shown in fig 1. To investigate whether the inhibition of cell proliferation attributes to apoptosis, gDNA fragmentation was assessed by gel DNA ladder formation on agarose gel as well as single cell-based comet assays in PC3. Cleavage/fragmentation of gDNA into oligonucleosomal size fragments is an integral part of apoptosis [24]. To investigate whether crude extract of leaf of OI indues autophagy in PC3, expression of autophagy marker genes; ATG1, ATG2 and LC3 were assessed by qPCR, However, no change in the expression of the maker genes was observed (data not shown). The dose-dependent gDNA fragmentation induced by the crude leaf extract observed in both DNA ladder formation and comet assays suggests that the extract induces cell apoptosis (fig 2).

Therefore, we developed a Bioassay Guided Fractionations (BGF) employing silica columnand the powerful preparative HPLC fractionations followed by a high throughput MTT assay as described above in materials and methods to purify and characterize the bioactive phytocompound from the leaf of OI that induces cells apoptosis (fig. 3A). The

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method of BGF has been into practice for the targeted purification of pharmaceutically active compounds from plant sources [25-30]. A single peak of the compound in the HPLC analyses of retention time 9.02 min showed the desired activity (fig. 3A). The IC_{50} value of oroxyquinone after purification was determined and found to be 19.44 µg/ml (95% CI = 17.97 to 21.04) as shown in fig 3B. The structure was elucidated based on spectroscopic techniques. The chemical characterization of the purified bioactive compound using NMR, FT-IR, and LC/MS reveal that the purified phytocompound is 2-[p-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol with the chemical formula of $C_{13}H_{18}N_2O_8$ (exact molecular mass = 330.11) and the chemical structure is as shown in fig. 4. Searching of 2-[p-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol using chemical databases namely SciFinder, ChemSpider, Pubchem, and Pubmed revealed that the purified phytocompound from OI in the current study is evidently a novel compound. Therefore, the purified bioactive compound is named (common name) as "Oroxyquinone" based on the source of purification (*Oroxylum indicum)* and the chemical structure (glycosylated hydro*quinone*) for convenience. Oroxyquinone induces cell cycle arrest at S phase in a dose-dependent manner in PC3 cells (fig. 5). Therefore, the findings clearly indicate that oroxyquinone triggers cell cycle arrest at S phase which may be possibly due to DNA fragmentation induced by the phytocompound. In view of the fact that the majority of these cancer-related deaths are due to tumor metastasis rather than to the primary tumors [31], we also investigated the anti-metastatic activity of oroxyquinone on PC3 cells which are highly metastatic *in vitro* using wound-healing assay. The results showed that oroxyquinone significantly inhibits the highly metastatic PC3 cells migration by approximately ≈35% at 6 hrs and ≈55% at 12 hrs. While additional migration and invasion test are needed in support, our findings suggest that oroxyquinone may also inhibits cancer cell migration.

Deciphering the molecular mechanism of oroxyquinone for apoptosis induction, activation of caspase-3, the executor caspase family member, was assessed in PC3 cells. Oroxyquinone does not activate caspase-3 protein in PC3 cells. Further, deactivation of caspase substrate PARP was assessed. As expected, oroxyquinone does not activate PARP also in PC3 cells (fig. 7). The results clearly indicate that oroxyquinone induces apoptosis in PC3 cells in a caspase-independent manner. Most cancer cells have a tendency to evade the classical process of apoptosis [32-34] and targeting alternate pathways to induce apoptosis to cancerous cells is a more recent development [32]. Determination of the molecular mechanism of apoptosis resulted in the understanding that PC3 cells treated with oroxyquinone could induce apoptosis in a caspase and PARP independent pathway. Our result supports previous studies carried out by various researchers [35-38] using phytochemicals as well as amino acids which suggest that PC3 cells have the potential to undergo both caspase-dependent as well as independent pathways in apoptosis. Further, involvement of Mitogen Activated Protein Kinases (MAPK) pathways was assessed by western blotting. MAPKs respond to a wide range of extracellular and intracellular changes. Stimulation of JNK and p38 trigger both anti-apoptotic as well as pro-apoptotic responses depending on the type of cell and stimulator [39]. It has been originally shown that ERKs are important for cell survival, whereas JNKs and/or p38 were deemed stress-responsive and involved in apoptosis. However, the regulation of apoptosis by MAPKs is more complex than initially thought and often controversial [40]. It has been reported that JNK activation triggers apoptosis in response to many types of stress including UV and radiation, protein synthesis inhibitors, and anticancer drugs (cisplatinum, adriamycin or etoposide). In most cases, p38 is simultaneously activated with JNKs [41]. The stimulation of p38 and JNK signaling pathways by oroxyquinone in PC3 cells in a dose-dependent manner suggests that cells require p38 and/or JNK activations for apoptosis induction. ERK is abruptly stimulated by oroxyquinone at high dose (40µg/ml) but not at low doses (10µg/ml and 20µg/ml) even though apoptosis is significantly induced at the low doses (Fig 8A). Therefore, it is likely that the ERK stimulation at the highest dose of oroxyquinone is not responsible for apoptosis induction in PC3 cells. To confirm the functional

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roles of p38, JNK, and ERK involved in the oroxyquinone induced apoptosis in PC3 cells, MTT assays were conducted in the presence and absence of specific inhibitors of p38, JNK, and ERK. The results revealed that the presence of the specific inhibitor of p38 (SB203580) abrogates the oroxyquinone induced inhibition of PC3 cell proliferation whereas the presence of specific inhibitors for JNK (SP600125) or ERK (U0126) enhances inhibition of PC3 cells proliferation (Fig 8B). Taken together, the findings strongly suggest that oroxyquinone induces apoptosis through p38 pathway and cell cycle arrest in prostate cancer cells. Activation of JNK by oroxyquinone may have other cellular effects in PC3 cells rather than apoptosis. However, a detailed investigation of the mechanism of the novel compound is warranted.

4. Materials and Methods

4.1 Cell Culture

PC3 cells were obtained from the National Centre for Cell Science (NCCS) Pune, India. Cells were cultured in RPMI 1640 (GIBCO, LifeTechnologies) supplemented with 10% FBS (GIBCO, LifeTechnologies), 1U PenStrep (GIBCO, LifeTechnologies) in cell culture plates. Cells were maintained in a humidified chamber with 95% atmospheric air and 5% CO_2 at 37°C.

4.2 Extraction and purification

The plant specimen was collected from Khurukhul, Manipur, India, and identified by the Botanical Survey of India, Eastern Regional Centre, Ministry of Environment and Forests, Shillong, India, and a voucher specimen was deposited under Voucher No. BSI/ERC/Tech/2016/463. Leaf of OI was collected, dried under shade, and grounded using a blender. Gross particles like midribs were removed before processing. Twenty-five grams of the leaf powder was weighed and subjected to aqueous extraction using a soxhlet apparatus (500 ml capacity) below 60°C for preliminary investigation of inhibition of cell proliferation. For purification of phytocompound, twenty-five grams of the leaf extract was weighed and subjected to hexane followed by chloroform extraction using a 500 ml capacity soxhlet apparatus. The crude extract was then condensed using a rotary vacuum evaporator followed by freeze-drying using a lyophilizer. The chloroform extract was subjected to fractionation using water at a final concentration of 50:50 Chl:H_2O and fractions were lyophilized. Lyophilized powders were used for bioassay for testing cells antiproliferative activity or stored at -20°C till used. The antiproliferative assays were tested on cultured cells using MTT assay as described below. A concentration of 100mg/ml of aqueous fraction was prepared in water for further purification using silica columnchromatography. Initial fractionation was conducted on a silica column chromatography (230-400 mesh, 40X600mm). 5ml of 100mg/ml crude extract was loaded in the column and fractions of 50ml were collected using water as mobile phase. Each fraction was tested for cytotoxic on cultured cells. Fraction showing bioactivity was further purified using HPLC (Agilent 1260 Infinity) on a Zorbax C-18 column, 5µm particle size 4.5X150mm column flow rate of 1ml per minute with 8% methanol 92% water in an isocratic mode for 10 min followed by a gradual increase in the percentage of methanol to 100% in 40 min. Each peak was collected in time slice mode for 1ml each and analyzed for cytotoxic activity. The peaks which showed significant bioactivity on cultured cells were collected and used for further assays.

4.3 MTT assay

Approximately 1×10^3 cells were seeded in a 96-well tissue culture plate. The cells were cultured in 100µl RPMI (without phenol red) containing 10% FBS, 5% CO_2 and incubated at 37°C. Cells were treated for 24, 48, and 72 hrs with 10µl of solution containing various doses of crude or purified compound in triplicates. The experiment was also conducted

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in the presence and absence of specific inhibitors for p38, JNK, and ERK. Control cells were treated with 10µl PBS under identical conditions as treated cells. At the end of treatment, 10µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5mg/ml) was added to each well and incubated at 37°C for 4 hrs. The MTT crystals formed were dissolved in 100µl of SDS-HCl solution and incubated for 4 hrs at 37°C. Numbers of viable cells were then quantified by measuring absorbance at 570nm on a microplate reader (Multiskan Go, Thermo Fisher Scientific, USA). The experiment was conducted three times in triplicate.

4.4 DNA fragmentation

DNA fragmentation assays were carried out as previously described [42]. Approximately 5×10^6 of PC3 cells were seeded in 6-well tissue culture plates and PC3 cells were treated with 50, 200, and 400µg/ml leaf extract of OI. Control cells were treated with DMSO as the negative control. Following treatment, cells were lysed using lysis buffer and subjected to electrophoresis on 2% agarose gel. The images were recorded on an imager (ChemiDoc, BioRad, USA).

4.5 Chemical characterization

The bioassay-guided purified compound was analyzed using an Agilent 6200 MS system. The purified compound was subjected to LC using 5% methanol and 95% water isocratic flow with proshell 120, C-18 column of 4.6 X 50 mm dimension and 2.7 µm particle size maintained at 30°C with a flowrate of 1ml per minute. The desired peak was selected and nebulized at 300°C, 40 psig, analyzed at 135v for a mass range between 100-1500 m/z.

All NMR spectra were recorded on Bruker-Avance 500 MHz spectrometer with TMS as an internal standard. ^{1}H chemical shifts are reported in delta (δ) units, in parts per million (ppm) downfield from tetramethylsilane, and also with reference to residual protic solvent (CDCl3, δH = 7.26 ppm). ^{13}C chemical shifts are referenced to the solvent signal (CDCl3). Infrared (IR) data were recorded as films on potassium bromide (KBr) pellets on a FT-IR spectrometer. Absorbance frequencies are reported in reciprocal centimeters (cm^{-1}). High-resolution mass spectra (HRMS) were performed on a VG Autospec-3000 spectrometer. The chemical structure and IUPAC name of the purified phytocompound was elucidated using ChemDoodle Software (iChemLabs, Ver. 9.0.3). To test whether the purified phytocompound is a new chemical compound, chemical databases namely SciFinder, ChemSpider, Pubchem, and Pubmed were used.

4.6 Comet assay

Comet assay was conducted as per the protocol described by Olive and Banath, 2006 [43]. Briefly, cells were treated with various doses of crude extract/HPLC purified compounds or PBS as a negative control for 24 hrs. Etoposide was used as positive control. Cells were trypsinized and approximately 5×10^3 cells were suspended in PBS, mixed with 1.2ml of low-melting agarose at 40°C by gentle pipetting, and poured on an agarose precoated microscope slide. After allowing solidification of the agarose at room temperature, the slide was submerged in neutral lysis solution (2% SDS, 0.5M EDTA, and 0.5mg/ml proteinase K) and incubated at 37°C for 16 hrs in the dark. The slides were then washed three times with neutral electrophoresis buffer followed by electrophoresis at 0.6V/cm for 25 min. Slides were then stained with propidium iodide (50µg/ml) and observed under a fluorescent microscope (Leica Microsystem) and images were recorded. The tail lengths of at least 25 comets in each slide were scored using Leica Application Suite (LAS), Leica Microsystem, GmBH, Germany software for analysis.

4.7 Wound Healing Assay

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Wound healing assay was conducted as described previously by Rodriguez and colleagues [44]. Cells were seeded in 6-well tissue culture plates, grown overnight, scratched the bottom surface with a sterile pipette tip, washed with PBS, and treated with 5µg/ml of the purified phytocompound. After treatments cells were observed in a microscope and pictures were taken at 0, 6, and 12hrs.

4.8 Western blots

Cells were seeded on 6-well tissue culture plates and treated with the purified compound or PBS (as a control) for 24 hrs. The cells were trypsinized, lysed, and equal amount of total proteins were separated by 10% SDS-PAGE. After transferring the separated proteins on the membrane, probed with anti-PARP, anti-caspase 3, anti-ERK, anti-P38, and anti-JNK antibodies. All antibodies used in the western blots were obtained from Cell Signalling, USA. The fluorescence signals were exposed to a photo film or directly recorded on an imager (Chemidoc, BioRad, USA).

4.9 Cell cycle analysis

Cells were seeded in 6-well tissue culture plates and treated with 10, 20, and 40µg/ml of purified plant compound for 16 hrs. After treatment, cells were harvested by trypsinized, washed with PBS, and fixed with 70% ethanol for 30 min at 4°C. The cells were washed with PBS, treated with 100µg/ml RNase, stained with propidium iodide (50µg/ml), and then cell cycle was analyzed on a flow cytometer (BD Acuri C5).

4.10 Statistical Analysis

Data obtained in the above studies were statistically analyzed and expressed as mean ± SD. Statistical significance was assessed by ANOVA followed by Tukey- Kramer multiple comparison tests using GraphPad Prism 8. The significance level was set at $P < 0.05$. Treated groups were compared with the untreated controls in MTT assay and comet assay. In the wound healing assay, treated groups were compared with the untreated controls of the same time points.

5. Conclusions

Cancer being a frontier area of research there has been significant development in the screening and discovery of potential anti-cancer drugs. We have purified a novel cytotoxic phytocompound, 2-[p-(2-Carboxyhydrazino)phenoxy]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol. Activation of MAPK pathways is required for apoptosis induced by oroxyquinone. In addition to the cytotoxic activity, oroxyquinone also has an anti-metastatic activity. India being a large nation with considerable potential in the development of nutraceuticals, the findings of the current study will be beneficial for the improvement of medicinal plants for use as therapeutic agents.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: FT-IR chromatogram of oroxyquinone, Figure S2: FT-IR chromatogram of oroxyquinone, Figure S3: NMR chromatograms of oroxyquinone.

Author Contributions: ARS, SAS, NTS, and TDS conducted the experiments. TCM analyzed the data and critically reviewed the manuscript. OKS analyzed chemical spectra. LSS conceived the idea and designed the experiments and wrote the manuscript. All the authors approved the final version of the paper.

Funding: This work was supported by the Department of Science and Technology (DST), Ministry of Science and Technology, Government of India [grant numbers: ERM/2015/001790] to LSS.

Informed Consent Statement: Not applicable

Acknowledgments: We thank Dr. Chaya Deori, Botanical Survey of India, Eastern Regional Centre, Ministry of Environment and Forests, Shillong, India for his valuable taxonomy identification of the specimen.

Conflicts of Interest: The authors have declared no conflict of interest.

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